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ORIGINAL ARTICLES

Assessment of Polymicrobial Infections in Ticks in New York State

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Abstract

Ixodes scapularis ticks are clinically important hematophagous vectors. A single tick bite can lead to a polymicrobial infection. We determined the prevalence of polymicrobial infection with *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia miyamotoi*, and Powassan virus in 286 adult ticks from the two counties in New York State where Lyme disease is endemic, utilizing a MassTag multiplex polymerase chain reaction assay. Seventy-one percent of the ticks harbored at least one organism; 30% had a polymicrobial infection. Infections with three microbes were detected in 5% of the ticks. One tick was infected with four organisms. Our results show that coinfection is a frequent occurrence in ticks in the two counties surveyed.

Key Words: Anaplasma—Babesia—Borrelia—Tick(s)—Diagnostic—Surveillance—MassTag PCR.

Introduction

Polymicrobial infections of ticks may result in transmission of several pathogens following a single tick bite. In the United States, the tick responsible for the majority of reported tick-borne infections is *Ixodes scapularis*. This tick is a vector of several human pathogens, most notably the bacteria *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, as well as the protozoan *Babesia microti* (Spielman et al. 1979, Burgdorfer et al. 1982, Pancholi et al. 1995). *B. burgdorferi* is the etiological agent of Lyme disease, the most common vector-borne disease in the United States with approximately 20,000 cases reported to the Centers for Disease Control and Prevention each year (Bacon et al. 2008). Infections with *A. phagocytophilum* result in approximately 500 reported annual cases of human granulocytic anaplasmosis (HGA) (CDC 2008). *B. microti* causes babesiosis, an erythrocytic infection of unknown prevalence due to it not being a reportable disease. In addition, several other important microbes can be transmitted by *I. scapularis*. Among these is Powassan virus, a flavivirus in the tick-borne encephalitis complex (Telford et al. 1997). Two distinct genotypes of Powassan virus exist: genotype II found in *I. scapularis* and genotype I found only in other Ixodid ticks (Ebel et al. 2001, Kuno et al. 2001). Although rare, Powassan encephalitis is nonetheless clinically important; only 40 cases have been diagnosed since its initial description in 1958; however, Powassan encephalitis has a case fatality rate of 10–15%. Further, the incidence may be increasing; seven cases were reported in 2007 (CDC 2001, Hinten et al. 2008). Along with *B. burgdorferi*, another *Borrelia* species, *Borrelia miyamotoi*, has been detected in *I. scapularis* (Fukunaga et al. 1995, Scoles et al. 2001). Genetically, *B. miyamotoi* is very similar to "*Borrelia lonestari*," a spirochete detected in *Amblyomma americanum* ticks (Fukunaga et al. 1996, Burkot et al. 2001). Phylogenetic analysis placed both *B. miyamotoi* and *B. lonestari* with *Borrelia* species associated with relapsing fever, although the pathogenicity of both species is unknown.

We recently described a multiplex method for rapid and economic screening of tick-borne pathogens (Tokarz et al. 2009). This method, MassTag polymerase chain reaction (PCR), enables efficient, sensitive screening of individual ticks for polymicrobial infection. Here we report its application for tick surveillance in Lyme disease endemic areas in New York State.

Materials and Methods

All PCRs were performed using cDNA generated from individual adult ticks. Live ticks were collected from vegetation in five separate locations in the two counties (Westchester and Suffolk) of New York State in the fall of 2008 (Fig. 1). Locations 1 and 2 are in Westchester County, whereas locations 3, 4, and 5 are in Suffolk County. Prior to nucleic acid extraction, ticks were immersed in 75% ethanol for 15 min, washed twice with phosphate-buffered saline, and then homogenized in Tri-reagent LS (Molecular Research Center, Cincinnati, OH). Total RNA was resuspended in 20 μL of

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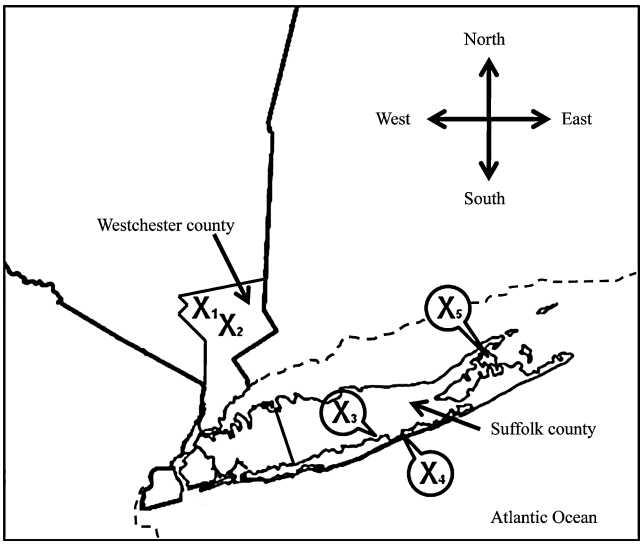

FIG. 1. Map indicating tick collection sites within New York State.

H_2O. cDNA was generated in a 20 μL reaction with Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) using 10 μL of total RNA. MassTag PCR was performed using the tick panel (Tokarz et al. 2009), with additional primers for the detection of Powassan virus and relapsing fever *Borrelia* species (Table 1). All MassTag PCR assays utilized 3 μL of cDNA. Reaction conditions were 95°C for 5 min, one cycle at 95°C for 20 s, 65°C for 20 s (annealing), 72°C for 30 s, followed by 11 cycles with annealing temperature decreased by −1°C at each cycle. The final PCR was run for 37 cycles at the annealing temperature of 54°C. For sequencing, PCR products were size-fractionated on ethidium bromide–stained 1% agarose gel, observed under ultraviolet illumination, excised, and gel purified for dideoxy sequencing.

Results

MassTag PCR was used to screen adult *I. scapularis* ticks collected from five locations in two different counties surrounding New York City (Fig. 1) for polymicrobial infection. The number of ticks screened from each location ranged from 24 to 98, with a total of 286 ticks analyzed. We detected at least one organism in 204 ticks (71%), of which 85 (30% of total) had a polymicrobial infection (Table 2). About 82 ticks (29%) did not have an infection with any of the microbes surveyed.

B. burgdorferi coinfection with A. phagocytophilum and B. microti

B. burgdorferi was the most common pathogen detected at all five sites, with infection rates of >50% ticks at each site (Table 3). Overall, we detected this organism in 182 out of the 286 ticks (64%). We detected *A. phagocytophilum* in >20% of the ticks at four out of the five sites; one notable difference being site 1, where only two out of the 56 ticks harbored the pathogen. Overall, a total of the 56 ticks (20%) were positive for *A. phagocytophilum*, of which 45 (80%) were also infected with *B. burgdorferi*. Conversely, 24% of the *B. burgdorferi*–positive ticks were also *A. phagocytophilum* positive. Ticks with a coinfection of *A. phagocytophilum* and *B. burgdorferi* accounted for 16% of total ticks analyzed. We detected *B. microti* in 58 ticks (20% of total), of which 48 were also infected with *B. burgdorferi* (83%).

B. burgdorferi coinfection with B. miyamotoi and Powassan virus

B. miyamotoi is a relapsing fever-like *Borrelia* species. This organism was much less frequently detected; only seven ticks (2%) were positive for this organism but all were also coinfected with *B. burgdorferi*. Infection with Powassan virus was also infrequent. We detected the virus in seven ticks (2% of total). Five of these were collected at location 2 and a single

Table 1. List of Primers in 5′ to 3′ Orientation

Pathogen/gene target	*Primer pair*
Powassan virus/*ns5*	Fwd CATCCGACCATGCACCTAGA
	Rev CCAAAGTGAGGATGTGTACCAAAG
Borrelia (relapsing fever)/*flaB*	Fwd GCTGAAGAGCTTGGAATGCAAC
	Rev GCAATTGCYTCATCCTGATTTG
[a]Powassan virus/*E*	Fwd GGCAACTGCATCTCTATRAATCC
	Rev CCTCATGCAGTGAAAATGGATATCTT
[a]*Anaplasma phagocytophilum/gltA*	Fwd GACAGGATCTTCTGGAGCAGGTT
	Rev GCTGGTGAACCAATCTCAGCAA
[a]*Borrelia burgdorferi/ospA*	Fwd GCGTTTCAGTAGATTTGCCT
	Rev TTGGTGCCATTTGAGTCGTA
[a]*Borrelia lonestari/flaB*	Fwd AGCACAAGCTTCATGGACATTGA
	Rev GAGCCGCTTGAACACCTTCTC
[a]*Babesia microti/18S rRNA*	Fwd GGGACTTTGCGTTCATAAAACGC
	Rev GCAATAATCTATCCCCATCACGAT
[a]*Francisella tularensis/16S rRNA*	Fwd CAGATGGATGAGCCTGCGTT
	Rev TTACACCGACTCCAACAGCTAGTACT
[a]*F. tularensis/iglC*	Fwd ATGATTATGAGTGAGATGATAACAAGAC
	Rev TGCAGCTGCAATATATCCTATTTTAGC

[a]Used for confirmatory polymerase chain reaction.
Fwd, forward; Rev, reverse.

Table 2. Summary of Pathogens Detected in All Five Surveyed Areas

Total 286 Ixodes scapularis	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Borrelia miyamotoi*	*Babesia microti*	Powassan virus
# of positive ticks (% of total)	56 (20%)	182 (64%)	7 (2%)	58 (20%)	7 (2%)

45 ticks with a *A. phagocytophilum* and *B. burgdorferi* coinfection
48 ticks with a *B. microti* and *B. burgdorferi* coinfection
14 ticks with a *A. phagocytophilum*, *B. microti*, and *B. burgdorferi* polyinfection
One tick with a *A. phagocytophilum*, *B. miyamotoi*, and *B. burgdorferi* polyinfection
One tick with a *A. phagocytophilum*, *B. microti*, *B. burgdorferi*, and *B. miyamotoi* polyinfection
82 ticks uninfected

Table 3. Frequency of Microbes Detected at Each Area

Location 1 56 Ixodes scapularis	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Borrelia miyamotoi*	*Babesia microti*	Powassan virus
# of positive ticks (% of total)	2 (4%)	33 (59%)	2 (4%)	3 (5%)	1 (2%)

Two ticks with a *A. phagocytophilum* and *B. burgdorferi* coinfection
Two ticks with a *B. microti* and *B. burgdorferi* coinfection
22 ticks uninfected

Location 2 98 Ixodes scapularis	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Borrelia miyamotoi*	*Babesia microti*	Powassan virus
# of positive ticks (% of total)	25 (26%)	67 (68%)	4 (4%)	21 (21%)	5 (5%)

22 ticks with a *A. phagocytophilum* and *B. burgdorferi* coinfection
17 ticks with a *B. microti* and *B. burgdorferi* coinfection
Five ticks with a *A. phagocytophilum*, *B. microti*, and *B. burgdorferi* polyinfection
One tick with a *A. phagocytophilum*, *B. miyamotoi*, and *B. burgdorferi* polyinfection
22 ticks uninfected

Location 3 83 Ixodes scapularis	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Borrelia miyamotoi*	*Babesia microti*	Powassan virus
# of positive ticks (% of total)	17 (20%)	54 (65%)	0	26 (31%)	1 (1%)

14 ticks with a *A. phagocytophilum* and *B. burgdorferi* coinfection
21 ticks with a *B. microti* and *B. burgdorferi* coinfection
Seven ticks with a *A. phagocytophilum*, *B. microti*, and *B. burgdorferi* polyinfection
20 ticks uninfected

Location 4 25 Ixodes scapularis	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Borrelia miyamotoi*	*Babesia microti*	Powassan virus
# of positive ticks (% of total)	7 (28%)	13 (52%)	0	6 (24%)	0

Four ticks with a *A. phagocytophilum* and *B. burgdorferi* coinfection
Six ticks with a *B. microti* and *B. burgdorferi* coinfection
One tick with a *A. phagocytophilum*, *B. microti*, and *B. burgdorferi* polyinfection
11 ticks uninfected

Location 5 24 Ixodes scapularis	*Anaplasma phagocytophilum*	*Borrelia burgdorferi*	*Borrelia miyamotoi*	*Babesia microti*	Powassan virus
# of positive ticks (% of total)	5 (22%)	15 (68%)	1 (5%)	2 (9%)	0

Three ticks with a *A. phagocytophilum* and *B. burgdorferi* coinfection
Two ticks with a *B. microti* and *B. burgdorferi* coinfection
One tick with a *A. phagocytophilum*, *B. microti*, and *B. burgdorferi* polyinfection
One tick with a *A. phagocytophilum*, *B. microti*, *B. burgdorferi*, and *B. miyamotoi* polyinfection
Seven ticks uninfected

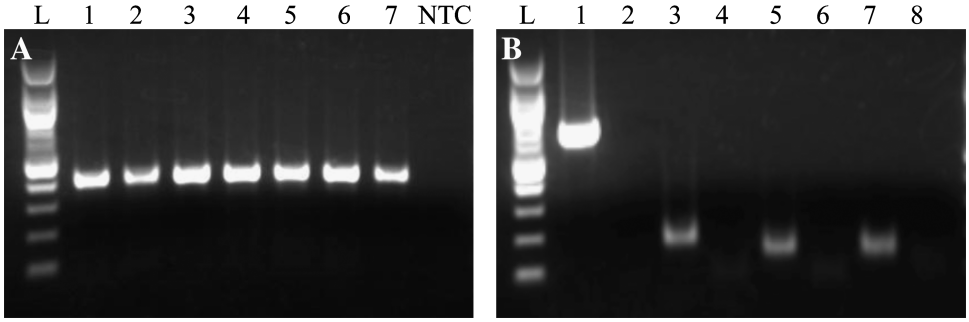

FIG. 2. Confirmation of MassTag polymerase chain reaction (PCR) results. (**A**) Powassan virus PCR using E gene primers on seven tick samples positive for Powassan virus by MassTag PCR (NS 5 gene; see Table 1). Lanes 1–7 represent tick samples, NTC, no template control; L, 100 base pair DNA ladder. (**B**) Confirmatory PCR of a polymicrobial infection in a single tick. Lane 1, *Borrelia burgdorferi ospA*; lane 3, *Babesia microti 18S rRNA*; lane 5, *Anaplasma phagocytophilum gltA*; lane 7, *Borrelia miyamotoi flaB*. Lanes 2, 4, 6, and 8 represent *ospA*, *18S rRNA*, *gltA*, and *flaB* no template controls, respectively.

Powassan virus–positive tick each was collected from locations 1 and 3. The majority of these did not exhibit a coinfection; only two of the Powassan virus–positive ticks were coinfected with *B. burgdorferi*, and another was coinfected with *A. phagocytophilum*, whereas the remaining four did not harbor any of the other infecting agents we screened for. For confirmation of Powassan virus–positive samples, we amplified by PCR and sequenced a 395-base pair fragment of Powassan virus (Fig. 2A) from each positive tick. All sequences represented genotype II of Powassan virus.

Triple infections

We detected ticks harboring a triple infection with *B. burgdorferi*, *A. phagocytophilum*, and *B. microti* at four sites. A total of 14 ticks (5% of total) had such a polymicrobial infection; we also detected a triple infection of *B. burgdorferi*, *A. phagocytophilum*, and *B. miyamotoi* in one tick. Additionally, we detected a single tick from area 5 infected with four pathogens (Fig. 2B).

Amblyomma and Dermacentor ticks

Along with *I. scapularis*, we also attempted to assess infection rates in other human biting ticks collected at two sites. We collected and screened 108 and 32 *A. americanum* ticks from locations 3 and 4, respectively. We detected *B. lonestari* in two ticks at each location. The only other organisms detected by our assay were *Ehrlichia* species, found in 17 ticks from area 3 and 2 ticks from area 4. Sequencing indicated that 10 ticks hosted *Ehrlichia ewingii*, 6 *Ehrlichia chaffeensis*, and a single tick the Panola Mountain *Ehrlichia* variant. Coinfections were not detected. Finally, we screened 13 *Dermacentor variabilis* ticks collected at area 4. We did detect *Francisella tularensis* in a single case. For confirmation, we amplified and sequenced fragments of the *IglC* and *16S rRNA* genes that were >99% similar to *F. tularensis* subspecies *tularensis*.

Discussion

We screened adult *I. scapularis* ticks collected from five different areas for pathogen infections. We found that 71% of the ticks were infected with at least one organism. In addition, we also detected a large number of coinfections; the number of ticks coinfected with at least two microbes was higher than the number of uninfected ticks. We also detected that 5% of the ticks were infected with triple infections and a single tick with four pathogens. Our results indicate that in the areas surveyed, coinfection of adult *I. scapularis* may be just as common as the lack of infection.

A. phagocytophilum and *B. microti* were each detected in 20% of the ticks. Surveillance studies indicated that the prevalence of these organisms in ticks varies greatly, though infection rates of >20% have been shown (Piesman et al. 1986, Schwartz et al. 1997, Schauber et al. 1998). High coinfection rates can increase the likelihood of multiple infections in humans following a single tick bite, a phenomenon already documented (Benach et al. 1985, Magnarelli et al. 1998, Belongia et al. 1999, De Martino et al. 2001, Krause et al. 2002). Lyme disease and HGA are the two most common diseases reported resulting from a bite of *I. scapularis*, although reported Lyme disease cases outnumber HGA by >30 to 1 (CDC 2008). In our analysis, tick infection by these pathogens was not dramatically different, with a ratio of approximately 3:1. Nearly a quarter of ticks infected with *B. burgdorferi* were also coinfected with *A. phagocytophilum*, implying that in the areas sampled, *B. burgdorferi* transmission may be accompanied by *A. phagocytophilum* transmission in up to 25% of the cases. Similar rates of coinfection with *B. burgdorferi* and *B. microti* also indicate high probability of cotransmission of these two organisms as well. The large discrepancy in HGA and Lyme disease cases may reflect failure in diagnosis, underreporting of HGA cases, or lower rates of *A. phagocytophilum* prevalence in some geographic areas. HGA may also be mild or subclinical. Further, little is known about the transmission dynamics of coinfecting microbes. In ticks hosting multiple pathogens, the microbial interaction may result in variable microbial loads, and such differences could lead to alteration in the transmission efficiency for a given microbe.

In addition, we screened for Powassan virus and *B. miyamotoi*, two microbes of much lower prevalence and not detected at all surveyed sites. Powassan virus has been reported to infect 1–5% of the ticks in the North Central United States, but prevalence studies of other areas are lacking (Ebel et al. 1999, Brackney et al. 2008). We detected Powassan virus and *B. miyamotoi* in 2% of the ticks we screened. The two microbes had different frequency of coinfection with other pathogens.

All seven *I. scapularis* infected with *B. miyamotoi* were also coinfected with *B. burgdorferi*. This is in contrast to Powassan virus–positive ticks, where in four out of the seven infected ticks we did not detect a coinfection. Whether this was a chance result or whether those ticks picked up the virus by feeding on an animal refractory to *B. burgdorferi* infection is unclear.

Polymicrobial infection following tick bites is an occurrence of which clinically much is still unknown. Our study indicates high coinfection prevalence in ticks within the areas surveyed. How these organisms interact during transmission and disease remains to be determined, but may have important impact on diagnosis and treatment of tick-borne diseases.

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Disclosure Statement

No competing financial interests exist.

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